15.

On the Relationship of Some Common Fishes as Determined by the Precipitin Reaction.

DOUGLAS G. GEMEROY
From the Zoological Laboratory, Rutgers University

(Text-figures 1-7).

INTRODUCTION.

It is generally agreed that the goal of taxonomy is the tracing of phylogenetic relationships and since the time of Darwin the emphasis in classification has been on the phylogenetic method in taxonomy. The chief source of data in phylogeny and classification has been morphological, and there is a need of some independent source of data with which to check the morphological interpretation. Such an independent source is available in the serological or biochemical constitution of animal proteins.

Nuttall's pioneer studies with the precipitin reaction applied to the determination of animal relationships have been followed by many others in both plants and animals, Mez and Ziegenspeck (1926); Landsteiner (1936) and others whose work is covered in the recent review of Boyden (1942). In the thirty-nine years since the publication of Nuttall's "Blood Immunity and Blood Relationship," progress has been made both in results and their interpretation, but the importance of serological data in taxonomy and phylogeny is still a controversial matter. In order to provide further data from which a conclusion might be drawn as to the relative importance of the serological method of attack on phylogeny and taxonomy, a study of the relationships of common fish was undertaken, using the old or common precipitin technique of the ring test and the new photron'er (photronreflectometer) methods introduced by Libby (1938). Such an approach is both quantitative and objective and is independent of morphology which it may complement and whose findings it may check. Further it may shed new light on many groups whose relationships are still uncertain with the methods available to morphology. The present paper represents the results of more than 500 tests upon the blood sera of 31 species of fresh water and marine fishes.

MATERIALS AND METHODS.

I. *Sources of Serum.*

The sera for the data of the present paper were procured from the species of fish listed below. Both common and scientific names are given.

Common Name	*Scientific Name*
Lamprey	*Petromyzon marinus* (Linnaeus)
Common Shark	*Carcharhinus* sp.
Yellow Shark	*Hypoprion brevirostris* Poey
Scyllium	*Scyllium canicula* (Linnaeus)
Dog-Fish	*Squalus acanthias* Linnaeus
Barn-door Skate	*Raja laevis* Mitchill
Eagle Ray	*Aetobatis narinari* (Euphrasen)
Sturgeon	*Acipenser rubicundus* (LeSueur)
Garpike	*Lepisosteus osseus* (Linnaeus)
Bowfin	*Amia calva* Linnaeus
Tarpon	*Tarpon atlanticus* (Cuvier and Valenciennes)
Brook Trout	*Salvelinus fontinalis* (Mitchill)
Rainbow Trout	*Salmo irideus* Gibbons
Brown Trout	*Salmo fario* Linnaeus
Carp	*Cyprinus carpio* Linnaeus
Buffalo Fish	*Ictiobus cyprinella* (Cuvier and Valenciennes)
Red Horse or Mullet	*Moxostoma aureolum* (LeSueur)

Catfish	*Ameiurus lacustris* (Walbaum)
Gaff-topsail Catfish	*Bagre marinus* (Mitchill)
Sea Catfish	*Galeichthys felis* (Linnaeus)
Pike	*Esox lucius* Linnaeus
Muskallunge	*Esox masquinongy* Mitchill
Barracuda	*Sphyraena barracuda* (Walbaum)
Dolphin or Dorado	*Coryphaena hippurus* Linnaeus
Black Bass	*Micropterus dolomieu* Lacépède
Large Mouth Bass	*Huro floridana* (LeSueur)
Rock Bass	*Ambloplites rupestris* (Rafinesque)
Blue Gill Sun Fish	*Helioperca incisor* (Cuvier and Valenciennes)
Perch	*Perca flavescens* (Mitchill)
Pickerel	*Stizostedion vitreum* (Mitchill)
Kingfish	*Menticirrhus americanus* (Linnaeus)

II. *Collection of Serum.*

The blood sera of the different fish listed above were obtained from various sources. The fresh water species were procured from Northern Ontario, the State Fish Hatchery at Hackettstown, New Jersey, the New York Aquarium and live fish markets in New York City. The salt water species were procured from a number of the different Biological Laboratories along the Atlantic and Gulf Coasts.[1]

III. *Handling of Serum.*

Two methods are used regularly to obtain blood from fish.

1. By syringe direct from the heart.
2. By drip method from the caudal artery.

With the fresh water species the first method was used throughout, while with the salt water species the second method was used in all cases except the shark, where the pericardial cavity was cut open and the blood allowed to drain into a receiving bowl directly. All bloods collected were allowed to clot for a period ranging from 6 to 12 hours. The serum that had been expressed from the clot by this time was poured off and centrifuged at 2,500 r.p.m. until free from cellular elements. In all cases where the serum was stored without any preservative it was first filtered through a Seitz filter and then bottled under sterile conditions. The greater portion of the sera collected however, was preserved by the addition of 0.02 ml. of 10 per cent. formalin to every ml. of serum. A few samples collected in the fall of 1940 were preserved by the addition of 1 ml. of 1:1000 merthiolate solution to each 10 ml. of serum. All the formolized sera were kept at room temperature during the course of the investigation, while the native and merthiolated sera were kept in the electric refrigerator, except when small samples were being used for antisera production or in carrying out the required tests.

IV. *Protein Determinations.*

Prior to the use of the different sera in antibody production, total nitrogen and non-protein nitrogen determinations were carried out by the micro-Kjeldahl digestion method of Koch and McMeekin (1929). From these figures the total protein was obtained by multiplying the T. N. — N.P.N. by factor 6.25. For N.P.N. determinations the protein was precipitated with trichloracetic acid. In the ring test where the end point of the reaction is the standard employed in determining relationships, comparable amounts of protein must be used in the different dilutions of the various antigens to give a quantitative basis for such relationships. In the tests reported here only standard and comparable antigen dilutions have been used.

The protein concentration of the fish sera in gms./100 ml. of serum varied from 1.50 gms. to 5.50 gms. The majority, however, were between 2.5 and 3.5 gms. The N.P.N. concentration in mgms./100 ml. of serum ranged from 20 to 125 mgms. with the exception of the elasmobranchs. Here the highest figures were recorded, being 1,000 mgms. or more in practically all members of this group.

V. *Injection Methods.*

Where antisera of maximum specificity are desired a single series of injections is administered. These injections are made on alternate days with the initial injection in all cases being 5 milligrams of protein per kilogram of body weight of the injected rabbit. Each subsequent injection is double the amount of the previous one until four injections in all are given. Where decreased specificity is desired in an antiserum to increase the range of cross reactions, multiple series of injections are required, Wolfe (1935). Preliminary tests with fish blood serum showed that the range of cross re-

[1] The author wishes to thank Drs. C. M. Breder, Jr. and Alan A. Boyden who contributed all the marine species used and Mr. C. O. Hayford, Superintendent of the State Fish Hatchery at Hackettstown, New Jersey, for a number of the fresh water species. He also wishes to express his appreciation to Drs. T. C. Nelson, C. M. Breder, Jr. and Alan A. Boyden for valuable aid and criticism of this paper. Submitted to the Graduate Faculty of Rutgers University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

actions in the majority of the antisera made to a single series of injections was rather restricted. To broaden the range of reaction multiple series of injections were employed with three rabbits. In multiple series injections, the initial series is followed after a lapse of one week by a second and even a third series of injections.

Eight days after the last injection, a trial bleeding of the animal was taken to determine whether the antiserum was of desired potency. In all but one case the antisera were sufficiently potent. On the following day the rabbits were anesthetized and all the blood obtainable was drawn directly from the heart by means of a large syringe and transferred to a finger bowl. The blood was allowed to clot in the refrigerator for eight to twelve hours, after which the expressed serum was decanted off, with minimum disturbance to the clot, and centrifuged at 2,500 r.p.m. until clear of cellular elements. The antiserum was then passed through a Seitz filter and bottled under sterile conditions. All the antisera thus treated were stored in the refrigerator at 4°C. ± 1°C. until needed. In no case where such a procedure was carried out did contamination occur in the refrigerated antisera.

VI. *Ring and Photron'er Tests.*

The ring and photron'er tests were carried out in the usual manner, the technique being that described by Boyden (1942).

Experimental Results.

The results of the different tests carried out by the ring and photron'er methods are shown in Tables I, II, III, IV. The percentage relationship found with the various sera used in the heterologous reactions is shown as a per cent. of the homologous tests with both ring and photron'er methods. With the ring test, duplicate readings were taken with all reactions, fresh dilutions of antigen and antisera being made up before the second series of tests was run. The antisera used in the ring tests were diluted 1:1 in all reactions, while with the photron'er they were used in full strength, as it was found in preliminary readings that diluting the antisera 1:4 eliminated practically all the heterologous reactions. Most of the antisera produced by means of a single series of injections were highly specific, hence no dilution was necessary.

Supplementing the tables are representative figures depicting homologous and heterologous reactions through their complete range as recorded on the photron'er, also representative graphs of the ring test showing the per cent. relationship values as determined from the titers of the homologous and heterologous antigens used.

Symbols.

A number of symbols are used in the tables and figures; the explanation of which is as follows:

(Fo)—Antigen was formolized. Where this symbol appears after an antiserum it means that the antiserum was produced by the use of a formolized antigen. In no case was the antiserum formolized.

(N)—Antigen was used without any preservative in all reactions. Antisera were produced with the native antigen.

(S3)—Antiserum produced by means of a triple injection series.

Discussion of Experimental Results.

I. *Technical Considerations.*

(a) *Preservation of antigens with formalin.*

As it is necessary to use some type of preservative when antigens are collected in the field, formalin was employed wherever such sera were preserved. From the unpublished data of M. A. Carriker, working in this laboratory, it was found that 0.2 per cent. formalin could safely be used for the field preservation of animal sera. In the present series of investigations, comparative tests were carried out to determine to what extent such treatment had altered the species specificity of the different blood sera. Text-figures 2 and 3 give an indication of the parallel results obtained when formolized and native antisera were reacted with the antigens used in their production. The same group of heterologous antigens show parallel relationship with the antinative and antiformolized antisera. Such results indicate that the use of formalin as a preservative under these conditions has not altered to any appreciable extent the "relationship" values shown by these two series of elasmobranch blood sera.

(b). *Comparison of photron'er and ring test methods.*

The ring test method of using the precipitin reaction in the determination of animal relationships has been the standard for many years and has been used successfully by many workers in serological investigations. With species that are not too closely related, this method is still valuable, as the range of reactions is great enough to include some distantly related species.

Although very sensitive at low concentrations of precipitated protein, the ring test as commonly performed gives only the end point, with no measure of the degree of reaction at intermediate levels. The photron'er, although less sensitive at the end point, gives a quantitative measure of the degree of reaction at every concentration of

the reacting substances. This instrument is far more effective than the ring test, therefore, where close relatives are involved. The ring test method can be likened to a low power microscope with its broad field of vision, while the photron'er acts like a much higher powered instrument. It pulls apart, as it were, antigens that show no difference in titer with the ring tests.

When the two methods are used in the precipitin reaction, one complements the other. Where rather widely separated species are being tested, the photron'er is, as a rule, highly specific and may not show any reaction in such cases. Where the ring test is employed and only the end point of the reaction is determined, the sensitivity of this method is apparent. By its use, cross reactions that can not be determined with the photron'er are established with the ring tests.

II. *Bearing of results on the problem of fish relationships.*

(a). *On animal relationships in general.*

As a fundamental approach in studying the diverse types of living organisms, the problem of the systematic relationship between such groups is of prime importance. The term animal relationship can be used to express any association between animals, but in its common usage it denotes systematic or genetic relationship. Although the principles on which animal relationships are based are by no means agreed upon by all taxonomists and morphologists, those outlined by Boyden (1942) seem to be the logical choice as a foundation for any particular attack on the problem of classification. Systematic relationships based upon conservative hereditary traits involve genetic relationship. To distinguish non-essential from essential criteria on which the grouping is based, should be the prime object. Those characters which most faithfully indicate a common heritage, will best serve as guides to the study of animal relationships.

Characters are not equal in this respect, however, for although the vast array of structural characters is primarily due to heredity in their mature expression, they may be affected by environment. By such interaction they may or may not help to reflect the common ancestry. Besides the morphological, both chemical and physiological characters are also determined primarily by inheritance. Some of these may also be modified in their mature expression by environmental influences. On the other hand, certain physiological characters, such as the blood groups, so far as is known, are determined so completely by inheritance as not to be affected by environment at all. On such a basis, it seems appropriate to use all kinds of conservative characters, morphological, biochemical and physiological, the choice of such characters depending on the relative constancy with which they indicate a common inheritance.

If it be true that the chemical nature of an organism, as well as its morphological expression, is determined primarily by inheritance, then morphology alone is not a complete basis for determining the degree of animal relationship. The evidence indicates that chemical similarity of proteins of various animal species denotes the relationship of such species, the closer the similarity of such proteins, the closer the degree of genetic relationship between them, Boyden (1942). The problem of classification is extremely complicated, but with a better understanding from the newer methods employed, it has much greater possibilities of achieving a truly natural classification than was formerly believed. In such a problem, then, we must be assured that the characters picked are conservative and any classification based on the nature of the organism should use all such characters and not confine itself to morphological expressions alone.

As a physiological character not appreciably affected by the environment to any extent, the blood sera of various animal species seem to be highly promising. Moreover, the conservativeness of this character is assured by means of the antibody mechanism existing in the organism, which apparently compels serum proteins to change but slowly. Since the discovery of the precipitin reaction by Kraus (1897) and the statements of Wells (1929) and Landsteiner (1936) that the chemical similarity of proteins can be demonstrated, many studies of the relationships of various animal species have been carried out using this method. Besides complementing in some cases the earlier findings of morphology, in others it has helped solve problems in classification that could not be worked out from the morphological evidence at hand, as shown in the work of Boyden and Noble (1933). Serological studies then can be an aid in helping to determine the degree of animal relationships.

(b.) *On the relationships between Cyclostomata, Elasmobranchii and Pisces.*

One very notable result in the attempt to demonstrate relationship between the above classes of Craniata by means of the serological method, was the marked inability of the antisera employed to show any cross reactions between these groups, even with the ring test. When antishark serum, produced by the triple injection method, was reacted with the lamprey (*Petromyzon marinus*) and the sturgeon (*Acipenser rubicundus*), no reaction whatsoever resulted (Table I). The inability to get any reaction between

Table I.

Ring and Photron'er Test Results of Elasmobranchs.

Antisera	Homologous Titer in Millions	Lamprey (Fo)	Shark 39 (Fo)	Shark 39 (N)	Shark 34 (Fo)	Shark 34 (N)	Hypodrion (Fo)	Hypodrion (N)	Scyllium (Fo)	Scyllium (N)	Dogfish (Fo)	Dogfish (N)	B.D. Skate (Fo)	B.D. Skate (N)	Eagle Ray (Fo)	Eagle Ray (N)	Sturgeon (Fo)
Ring Tests																	
Anti-Shark (S3) (Fo)	1.024	0.0	100		50		50		25		12.5		6.25		0.0		0.0
Anti-Shark I (Fo)	.256	0.0	100	100	100		50		12.5		0.0		0.0		0.0		
Anti-Shark II (N)	1.024		25	100	100			50		12.5		.78		0.0		0.0	
Photron'er Tests																	
Anti-Shark (S3) (Fo)		0.0	100		94		77.9		18.7		8.2		0.0				0.0
Anti-Shark II (N)				100		90.9		56		12.9		0.0					

these three classes is an indication of how far apart they really are. These serological differences point to a long evolutionary period during which the serum proteins of these classes have been evolving. The primary object of any classification is to put like things together and to separate the unlike. On this basis, then, there does not seem to be any justification for placing three such widely separated groups, as determined by serological methods, into one class and designating each as a sub-class of the larger group. Measured by our serological "yardstick" the Cyclostomata, Elasmobranchii and Pisces are really distinct classes.

(c.) *On the relationships within the classes of fishes.*

Intra-class variations of serum proteins would not be expected to be as great as between classes. Such intra-class variations do exist, nevertheless, to nearly the same degree as between classes when their serum proteins are compared. As an example, only with antishark serum produced with triple injections was any reaction found between the shark (*Carcharhinus* sp.), the dog-fish (*Squalus acanthias*) and the barn door skate (*Raja laevis*). No reaction whatever with this antishark serum could be shown when tested with the eagle ray (*Aetobatis narinari*) (Table I). The absence of any cross reaction between these two groups indicates relatively distant relationship between these orders of the same class.

Such a result indicates that the sharks and rays have also had a long period of evolutionary divergence. It seems clear then that orders and classes may have very different values as regards their degree of relationship when different vertebrate groups are being discussed. DeFalco (1940) working with birds had no difficulty in showing reactions between the most widely separated orders of this group with serological methods. A further example of the distances which may separate fishes of a group, was the result found with the ganoids, sturgeon (*Acipenser rubicundus*), garpike (*Lepisosteus osseus*) and bowfin (*Amia calva*). The differences here were not as wide, however, as found with the species of Elasmobranchii tested. Triple series antisturgeon serum gave but small reaction with the garpike and bowfin. When a single series antisturgeon serum was tested with these two species, no reaction was recorded, even though with the homologous antigen the potency of the single series antiserum was much greater than that of the antisturgeon produced by the triple series injection.

(d). *Results which parallel the present classification.*

In the more closely related groups where antigens were available, the serological

TABLE II.

Results of Ring Tests in Relation to Titers Homol-Titer = 100%.

ANTIGENS

ANTISERA	Homol. Titer in Millions	Sturgeon (Fo)	Gar-Pike (Fo)	Bowfin (Fo)	Bowfin (N)	Tarpon (Fo)	Tarpon (N)	Brook Trout (Fo)	Brook Trout (N)	Rainbow Trout (N)	Brown Trout (N)	Carp (Fo)	Carp (N)	Buffalo Fish (N)	Red Horse (N)	Catfish (Fo)	Pike (Fo)	Barracuda (Fo)
Anti-Sturgeon (S3) (Fo)	2.048	100	12.5	1.56		0.0		0.0				0.0				0.0	0.0	
Anti-Sturgeon I (Fo)	4.096	100	0.0	0.0		0.0		0.0				0.0				0.0		
Anti-Sturgeon II (Fo)	2.048	100	0.0	0.0		0.0		0.0				0.0				0.0		
Anti-Bowfin I (Fo)	.512	0.0	0.0	100	100	0.0		0.0				0.0				0.0	0.0	0.0
Anti-Bowfin II (N)	2.048	0.0	3.12	50	100		0.0		0.0				0.0			0.0	0.0	
Anti-Tarpon I (Fo)	1.024		0.0	0.0		100	100	0.0				0.0				0.0		
Anti-Tarpon II (Fo)	.032					100	100	0.0					0.0					
Anti-Brook Trout I (N)	.512				0.0		0.0	100	100	100	50		3.12			0.0	.20	
Anti-Brook Trout II (N)	.256						0.0	50	100	50	50		0.0		3.12	0.0	0.0	
Anti-Carp (Fo)	.512			0.0		0.0						100	100	6.25	12.5	0.0	0.0	
Anti-Carp I (N)	.512				.20		0.0		0.0			100	100	12.5	12.5	12.5	0.0	
Anti-Carp II (N)	.256				1.56		0.0		0.0			25	100	12.5	12.5	25	0.0	

ANTIGENS

ANTISERA	Homol. Titer in Millions	Bowfin (Fo)	Tarpon (Fo)	Brook Trout (Fo)	Carp (Fo)	Catfish (Fo)	Pike (Fo)	Muskallunge (Fo)	Barracuda (Fo)	Dolphin (Fo)	Black Bass (Fo)	L. M. Bass (Fo)	Rock Bass (Fo)	B.G.S. Fish (Fo)	Perch (Fo)	Pickerel (Fo)	Kingfish (Fo)
Anti-Pike I (Fo)	.512	0.0	0.0	1.56	0.0	3.12	100	25	0.0	.39	0.0					0.0	
Anti-Pike II (Fo)	.256		0.0	0.0	0.0	.39	100	50	0.0	0.0	0.0	0.0				0.0	
Anti-Muskallunge I (Fo)	.256			0.0		0.0	100	100	0.0	0.0	0.0	0.0			0.0	0.0	
Anti-Muskallunge II (Fo)	.256					0.0	50	100	0.0	0.0	0.0	0.0			0.0	0.0	
Anti-Barracuda I (Fo)	.256					0.0	0.0	0.0	100	0.0	.78				0.0		
Anti-Black Bass I (Fo)	.512					0.0			.39	0.0	100	50	25	25	6.25	3.12	0.0
Anti-Black Bass II (Fo)	1.024	0.0	0.0	1.56	0.0		0.0	0.0	1.56	1.56	100	50	50	50	6.25	3.12	0.20
Anti-Pickerel I (Fo)	.512			0.0			0.0	0.0		.78	3.12	6.25	1.56	6.25	6.25	100	0.0
Anti-Pickerel II (Fo)	.512			0.0				0.0	0.0	0.0	12.5	12.5	6.25	25	50	100	0.0

TABLE III.

Photron'er Results in Relation to Curve Area Homologous Curve=100%.

ANTISERA	Shark (39) (Fo)	B.D. Skate (Fo)	Eagle Ray (Fo)	Sturgeon (Fo)	Gar Pike (Fo)	Bowfin (Fo)	Bowfin (N)	Tarpon (Fo)	Tarpon (N)	Brook Trout (Fo)	Brook Trout (N)	Rainbow Trout (N)	Brown Trout (N)	Carp (Fo)	Carp (N)	Buffalo fish (N)	Redhorse (N)	Catfish (Fo)	Pike (Fo)
Anti-Sturgeon (S3) (Fo)	0.0		0.0	100	4.6	0.6		0.0		0.0				0.0				0.0	0.0
Anti-Sturgeon I (Fo)		0.0		100	0.0	0.0		0.0											
Anti-Sturgeon II (Fo)			0.0	100	0.0	0.0								0.0					
Anti-Bowfin (N)				0.0	7.0	63.8	100											2.3	
Anti-Bowfin (Fo)				0.0	0.0	100	88.6	0.0		0.0				0.0				0.0	
Anti-Tarpon I (Fo)						0.0		100		0.0				0.0					
Anti-Trout I (N)									0.0		100	68	60.3		0.0				
Anti-Trout II (N)							0.0		0.0	78.6	100	66	53.2		0.0			0.0	
Anti-Carp I (N)											0.0				100	6.8	9.3	0.0	
Anti-Carp II (N)															100	0.0	0.0		
Anti-Carp (Fo)														100		2.0	2.5		

ANTISERA	Brook Trout (Fo)	Carp (Fo)	Catfish (Fo)	Pike (Fo)	Musallunge (Fo)	Barracuda (Fo)	Black Bass (Fo)	L. M. Bass (Fo)	B.G.S. Fish (Fo)	Rock Bass (Fo)	Perch (Fo)	Pickerel (Fo)
Anti-Pike I (Fo)	0.0	0.0	0.0	100	15.1	0.0						
Anti-Pike II (Fo)			0.0	100	35.6	0.0						
Anti-Muskallunge I (Fo)			0.0	19.1	100	0.0						
Anti-Muskallunge II (Fo)			0.0	20.3	100	0.0						
Anti-Barracuda I (Fo)				0.0		100	0.0					
Anti-Black Bass I (Fo)							100	45.1	41.2	13.1	0.0	0.0
Anti-Black Bass II (Fo)	0.0						100	74.6	56.8	30.7	4.9	4.6
Anti-Pickerel I (Fo)							3.0				12.7	100
Anti-Pickerel II (Fo)	0.0						3.2	11.5		4.0	22.0	100

Table IV.

Ring and Photron'er Test Results of Catfish.

Antisera	Homologous Titer in Millions	Eagle Ray (Fo)	Sturgeon (Fo)	Gar Pike (Fo)	Bowfin (Fo)	Tarpon (Fo)	Brook Trout (Fo)	Carp (Fo)	Catfish (Fo)	Gaff Topsail Catfish (Fo)	Sea Catfish (Fo)	Pike (Fo)	Muskallunge (Fo)	Barracuda (Fo)	Dolphin (Fo)	Blackbass (Fo)	Perch (Fo)	Pickerel (Fo)
								Antigens										
							Ring Tests											
Anti-Catfish (S3) (Fo)	2.048	0.0	6.25	6.25	6.25	0.0	3.12	3.12	100	50	50	6.25	6.25	0.0	0.0	0.0	0.0	0.0
Anti-Catfish II (Fo)	.256		0.0	0.0	0.0		0.0	0.0	100	25	12.5	0.0	0.0		0.0	0.0		
							Photron'er Tests											
Anti-Catfish (S3) (Fo)			0.0		7.7		1.6	2.0	100	15.0	14.7	20.1		0.0		0.0		
Anti-Catfish II (Fo)					0.0			0.0	100			0.0						

findings in most cases parallel those established by the methods of morphology. This is best shown with the orders and families within the Teleostei. Cross reactions (photron'er) were found when antibrook trout (*Salvelinus fontinalis*) serum was tested with rainbow trout (*Salmo irideus*) and the brown trout (*Salmo fario*). No cross reaction could be shown with antibrook trout serum against carp, bowfin, catfish and tarpon. The pike (*Esox lucius*) and the muskallunge (*Esox masquinongy*) gave good cross reactions with this instrument, but very little reaction was demonstrated even with the ring test with 7 and 8 other species respectively, as shown in Table II.

In the group Percoidea of the family Acanthopteri, the antiblack bass (*Micropterus dolomieu*) and the antipickerel (*Stizostedion vitreum*) sera gave excellent cross reactions with closely related species (Table III). With the ring test, as would be expected, slight cross reactions were shown between species somewhat more distantly related (Table II).

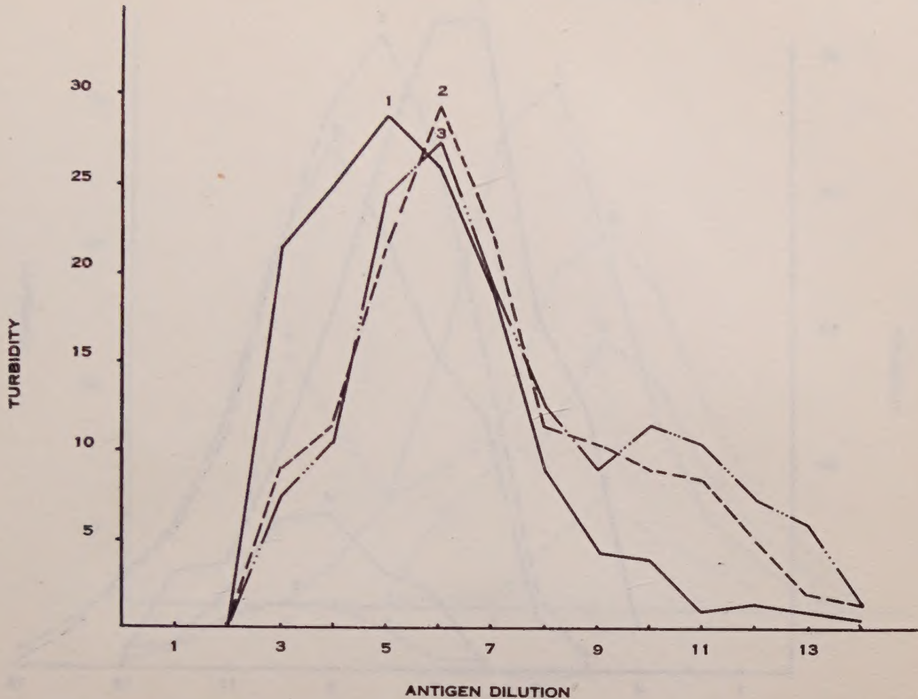
These results as determined by the serological method, could no doubt, be enlarged when more extensive studies are carried out with other species shown to be closely related by morphological methods.

(e). *Results which do not parallel the present classification.*

As just stated, the majority of the tests carried out gave relationships that paralleled the systematic position as determined by morphological methods. A small number of apparent discrepancies was observed and further studies will have to be carried out before one can be sure that the relationships shown are valid.

The cross reaction between the catfish (*Ameiurus lacustris*) and the bowfin indicates a 7.7% relationship which is surprising when compared with a 2.0% for catfish-carp and 1.6% for catfish-trout. Carp and trout are placed much closer to the catfish in their systematic position. On this basis a relationship of less than 1.6% was to be expected instead of the 7.7% obtained. Table IV.

The antiserum used in these reactions was produced by a multiple series of injections. Such antisera have been shown to have a broader range and a decreased specificity (Wolfe, 1935). Cross reactions may be produced when such antisera are tested with distantly related species that do not give a true indication of the relationships of these species. This is particularly important when the values obtained are small. With a catfish antiserum, produced by means of a series of single injections, no reaction was found with either bowfin, carp or trout. One single series bowfin antiserum, however, in the reciprocal reaction when cross reacted with the catfish, gave a relationship of 2.3%,



TEXT-FIG. 1. The comparative values when three different native carp antigens were tested against the same antinative carp serum. Relationships are determined by comparison of total areas of turbidity curves. Although the curves are not identical, the areas of these curves vary less than 2 per cent.

Antiserum	*Antigen*	*Curve No.*	*Per Cent. Area*
Anticarp 2 ×	Carp 1 (N)	1	99.5
	Carp 2 (N)	3	100.0
	Carp 3 (N)	2	98.3

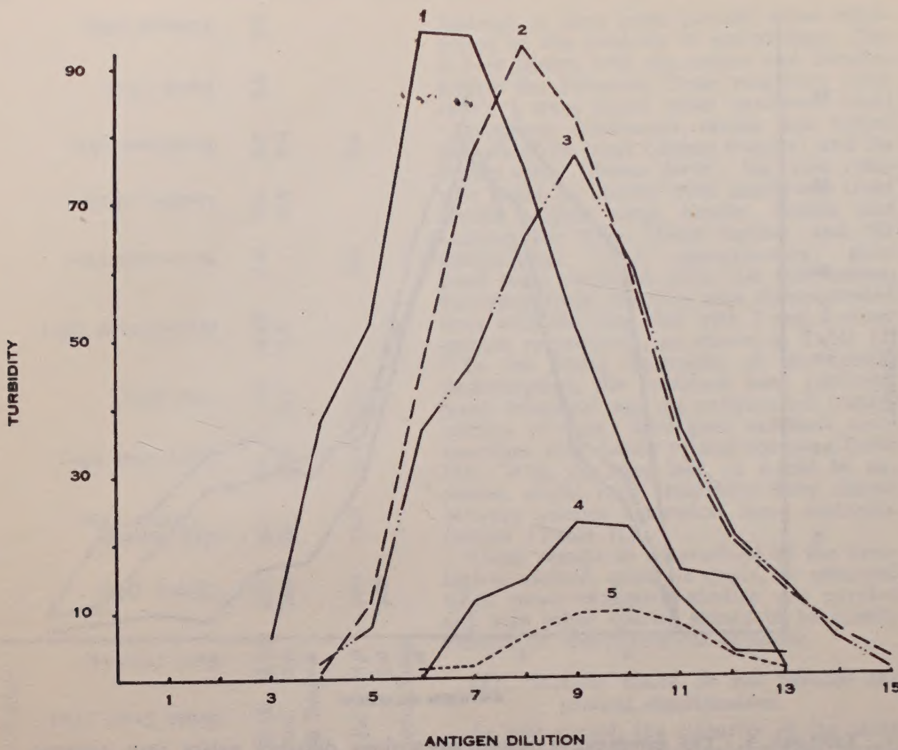
while another showed no reaction with either catfish, carp or trout. The same multiple series catfish antiserum gave a slightly stronger reaction with the pike than it did with two species of marine catfish.

Another result showing an apparent discrepancy with the present systematic position, was the tarpon (*Tarpon atlanticus*) classed as one of the most primitive of the Isopondyli. If any cross reactions with the ganoid antisera could be obtained, the tarpon, as one of the most primitive species of the Teleostei, should show such reaction. No reaction occurred between antibowfin serum and tarpon antigen, nor did antitarpon serum react with bowfin antigen. Table II. These few apparent exceptions to the general parallelism, require confirmation but do not seriously detract from the significance of the results.

(f). *Possible sources of error in serological studies.*

Careful consideration should be given to sources of error that may have a bearing on the analysis of any experimental results obtained. In serological investigations, the effects of lipoids on cross reactions have been studied (Landsteiner, 1936). It has been shown that by the addition of lipoids, cross reactions can be greatly increased. Although lipoids may not be capable of acting as antigens in antibody production, they can combine with serum proteins and act as haptenes and thus decrease the specificity of the reaction.

When the ring test is used in the precipitin reaction for the determination of animal relationships, comparable amounts of antigen and antibody must be used, as only the end point of the reaction is recorded in such

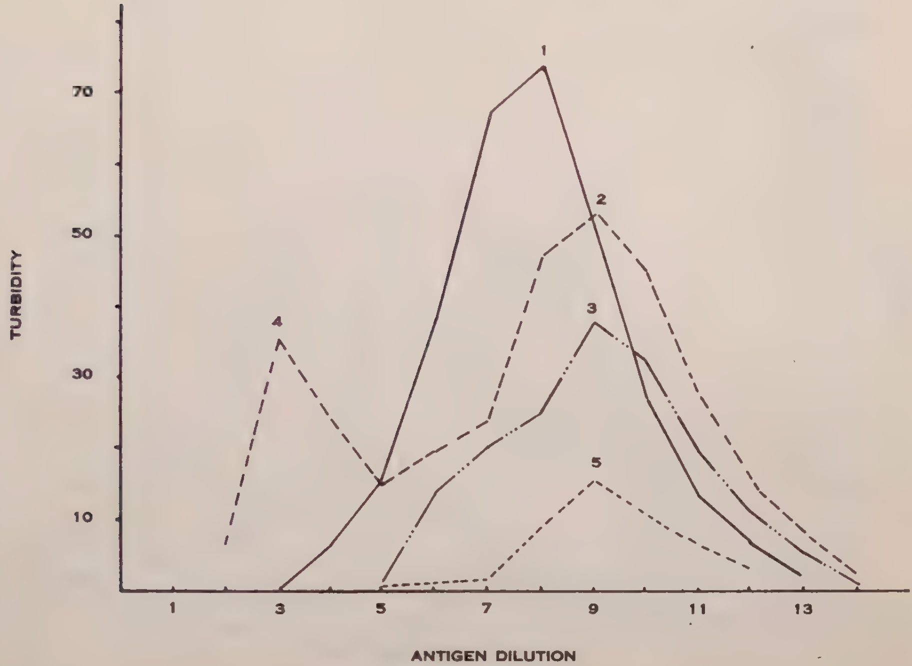


TEXT-FIG. 2. The relationship between different species of Elasmobranchii. This antishark serum was produced by triple injection series of a formolized antigen and only by such means could a reaction be shown with the dogfish. Formolized lamprey, barn door skate and sturgeon did not react with this antiserum.

Antiserum	*Antigen*	*Curve No.*	*Per Cent. Area*
Antishark (S3) (Fo) ×	Shark I (Fo)	1	100.0
	Shark II (Fo)	2	94.1
	Yellow shark (Fo)	3	77.9
	Scyllium (Fo)	4	18.9
	Dogfish (Fo)	5	8.2

determinations. With the photron'er, where the complete range of reaction is represented by the area under the curve, no such procedure is necessary. Where sufficient amounts of antigen and antibody are present, the reaction range is always complete. With constant antibody, the curve area remains the same, for as the amount of the antigen varies, the position only of the curve on the abscissa is changed.

As a rule serum proteins are not homogeneous substances and it is of prime importance to determine, if possible, just what fractions of the serum proteins are most active in antibody production and titration. The amounts and proportions of these proteins may vary in different species and such variation directly affects the antibody produced, which in turn affects the reaction when various antigens are reacted with antiserum. It is especially true that in the ring test, errors may result from comparison of the sera of different species, equivalent in total protein, but different in their content of active protein antigen. On the other hand, in the photron'er comparison with the same sera, no similar errors could occur if complete curves are obtained. In this respect, DeFalco (1940) has made an excellent beginning with the blood serum of birds. He found a wide difference in the albumin globulin ratios of certain birds and



TEXT-FIG. 3. The relationships between the Elasmobranchii with an antishark serum made by a single injection series. The antigen used for anti-serum production was not formolized as with the previous one. Text-figures 2 and 3 show the parallel results obtained with the same species, Text-figure 2 involving only formolized reagents, Text-figure 3, involving only native reagents.

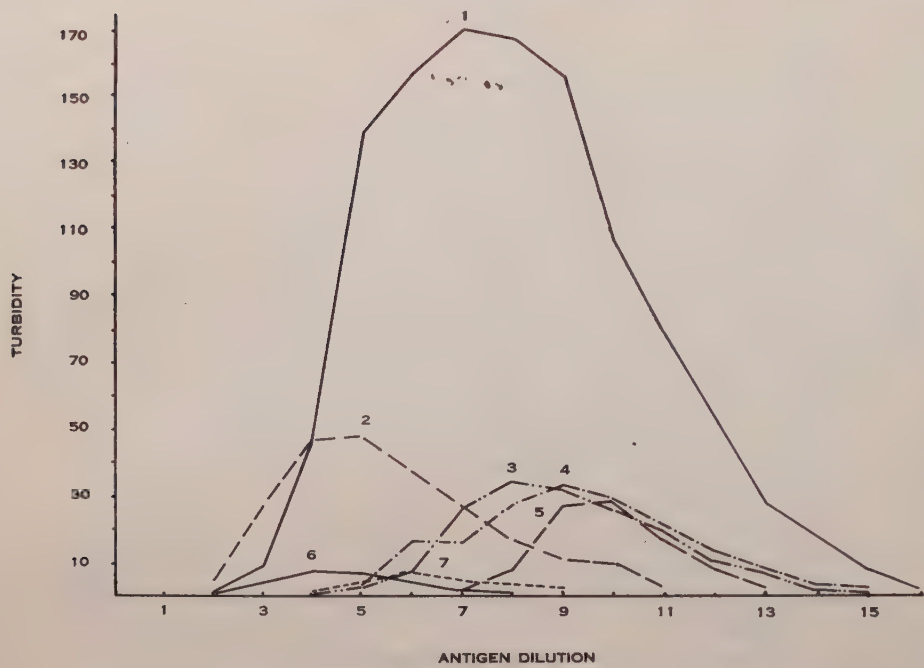
Antiserum	*Antigen*	*Curve No.*	*Per Cent. Area*
Antishark (N2) ×	Shark I (N)	1	100.0
	Shark II (N)	2, 4	90.9
	Yellow Shark (N)	3	56.0
	Scyllium (N)	5	12.9

demonstrated that the globulin fraction of blood serum is by far the most active of the proteins in antibody production and titration. Further investigation may show that it is necessary to compare only specific fractions of blood serum and not antigenic mixtures, such as the sera of birds and mammals. It does not necessarily follow that the effects found with birds can be applied to other classes of Craniata, the sera of which may be more nearly constant in their albumin globulin ratios.

If reactions are produced with very distant relatives when a multiple series antiserum with decreased specificity is employed, they should be checked before being accepted. This can be accomplished by dilution of the antiserum. By this means only related species show any cross reactions, thereby eliminating any that may seem doubtful.

(g). *General conclusions and problems for future study.*

The data presented in this paper seem to justify the conclusion that the precipitin reaction can be of value as a check on the morphological findings in the study of animal relationships. In most cases these findings parallel the systematic position of the several fishes, especially where closely related species are tested. In other cases they show apparent divergence from the usual systematic arrangement. An examination of some of the more recent classifications of fish, constructed entirely from the morphological approach, reveal the wide range in ideas held by taxonomists in general as to what principles should be employed as a foundation for determining the phylogenetic relationship of this rather

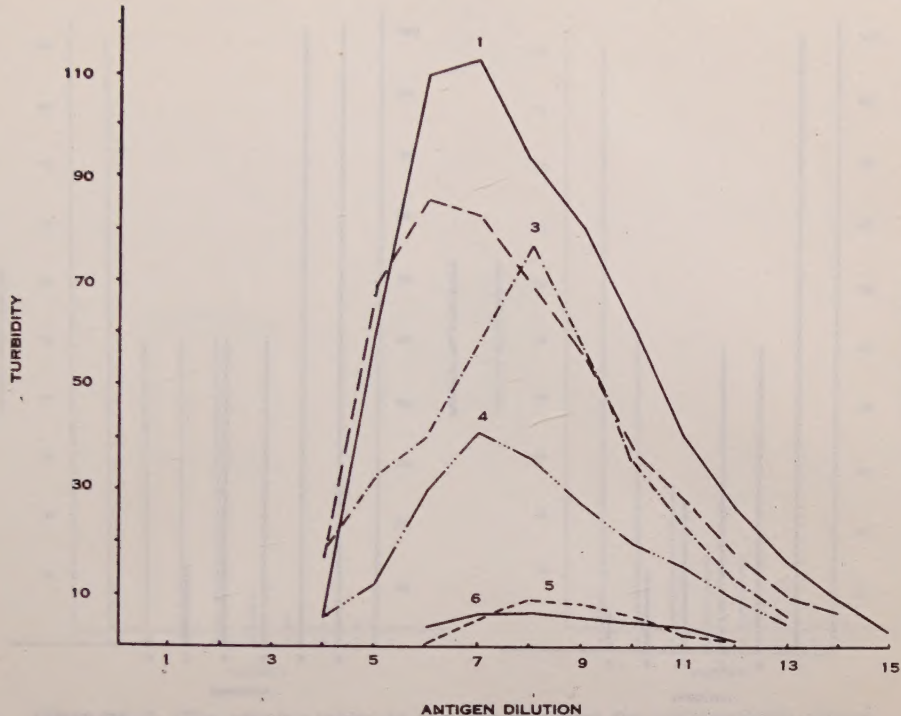


TEXT-FIG. 4. The reaction of antiformolized catfish serum produced by triple series method. The reactions with the formolized antigens indicated here are quite unexpected as enlarged upon in the discussion. Also it may be noted that pike antigen gave a stronger reaction than did either of the marine catfish, and further, the pike cross reaction was stronger than with the carp which is classed in the same order as the catfish. No reactions were produced with formolized barracuda, black bass or sturgeon antigens.

Antiserum	*Antigen*	*Curve No.*	*Per Cent. Area*
Anticatfish (S3) (Fo) ×	Catfish (Fo)	1	100.0
	Pike (Fo)	2	20.2
	Gaff Topsail (Fo)	3	14.7
	Sea Catfish (Fo)	4	15.0
	Bowfin (Fo)	5	7.7
	Carp (Fo)	6	2.0
	Brook Trout (Fo)	7	1.6

highly diversified group. Garstang (1931) states that recent classifications can hardly be taken as an expression of their phylogeny. In any case, a truly natural classification must take into account all the evidence discernable which bears upon the phylogeny. Only by so doing can a classification with probably correct genetic implications result. Because the serological and the morphological methods do not always agree it does not necessarily mean that the one is entirely right and the other entirely wrong. No phylogenetic method is final, be it morphological or serological. In the absence of certain knowledge of phylogeny we are forced to accept the more probable interpretations of animal relationship and these more probable interpretations must be based on more than one kind of evidence. In a real sense morphology and serology must complement each other.

There is, therefore, a need for further intensive studies among widely separated groups of fish to determine by serological methods what relationships can be shown between orders, families and species. The possible sources of error in the serological method as enumerated in (f) should be thoroughly investigated and their effects noted, so that correct conclusions may be drawn. With more nearly complete knowledge at our disposal, it may be possible to



TEXT-FIG. 5. The reaction values between representative species of the group Percoidea. As with the trout species tested the closely related species here are readily differentiated by the photron'er. The representatives of the family Percidae, the perch and the pickerel, seemingly are not closely related to the family Centrarchidae, even though placed in the same group. The black and large mouthed bass, two closely related species, are easily distinguished by the photron'er.

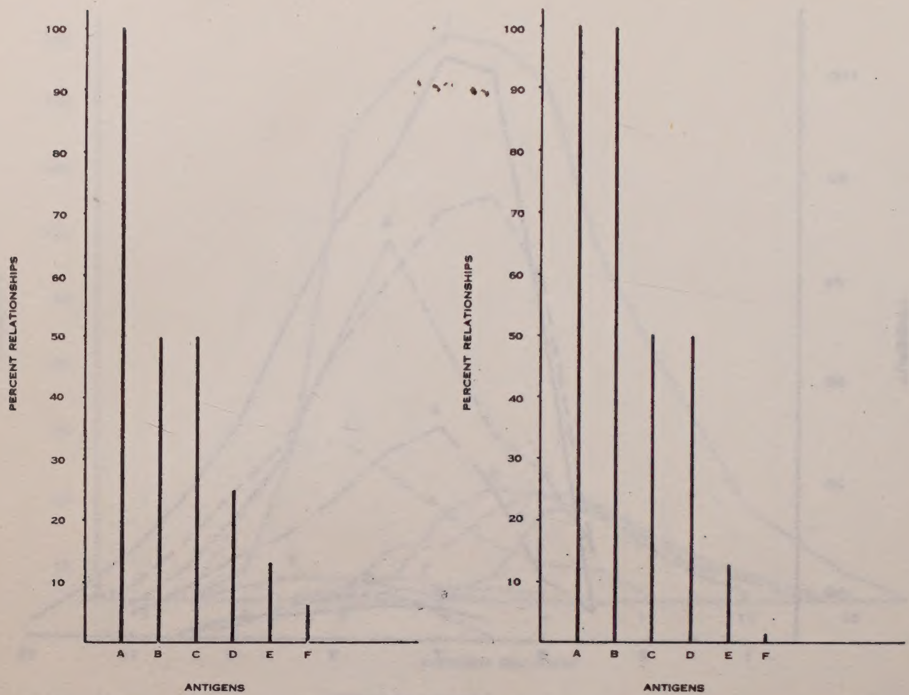
Antiserum	*Antigen*	*Curve No.*	*Per Cent. Area*
Antiblack Bass II (Fo) ×	Black Bass (Fo)	1	100.0
	L. M. Bass (Fo)	2	74.6
	B. G. Sunfish (Fo)	3	56.8
	Rock Bass (Fo)	4	30.7
	Perch (Fo)	5	4.9
	Pickerel (Fo)	6	4.6

understand the real significance of the serological methods, especially the precipitin reaction in the determination of the relationships between the different species of fish.

It may be that a quantitative basis for the determination of phylogenetic relationships can be established by the precipitin reaction. Such a serological "yardstick," besides its important application in classification, might be of great value in the field of animal breeding and thus mark out new lines of progress in practical genetics.

SUMMARY.

1. The precipitin test has been applied to the comparison of the blood sera of 31 species of fresh and salt water fishes. In all 43 antigens and 26 antisera were used in these tests, which were of two types, the ring test and the photron'er test.
2. Formolized antisera when tested with formolized antigens give parallel values with native antisera tested with native antigens.



TEXT-FIG. 6. The comparative values by the ring test of the Elasmobranchii tested against antiformolized and antinative shark sera. It will be observed that the barn door skate gave a slight reaction when tested against the antiformolized shark serum produced with the triple series method, while none was shown with an antiserum to a single series.

Antishark (S3) (Fo)
× A — Shark I (Fo)
B — Shark II (Fo)
C — Yellow Shark (Fo)
D — Scyllium (Fo)
E — Dogfish (Fo)
F — B. D. Skate (Fo)

Antishark (N2)
× A — Shark I (N)
B — Shark II (N)
C — Yellow Shark (N)
D — Shark I (Fo)
E — Scyllium (N)
F — Dogfish (N)

3. Cyclostomata, Elasmobranchii and Pisces, serologically are sufficiently far apart to be considered distinct classes.
4. Within the Elasmobranchii, antishark serum produced no cross reactions with the rays employed. Only one slight reaction was obtained with the skate.
5. In general the results parallel the taxonomic position based on morphology, but it is clearly evident that the chemical gulf which separates species and orders among fishes is far wider than that in birds.

BIBLIOGRAPHY.

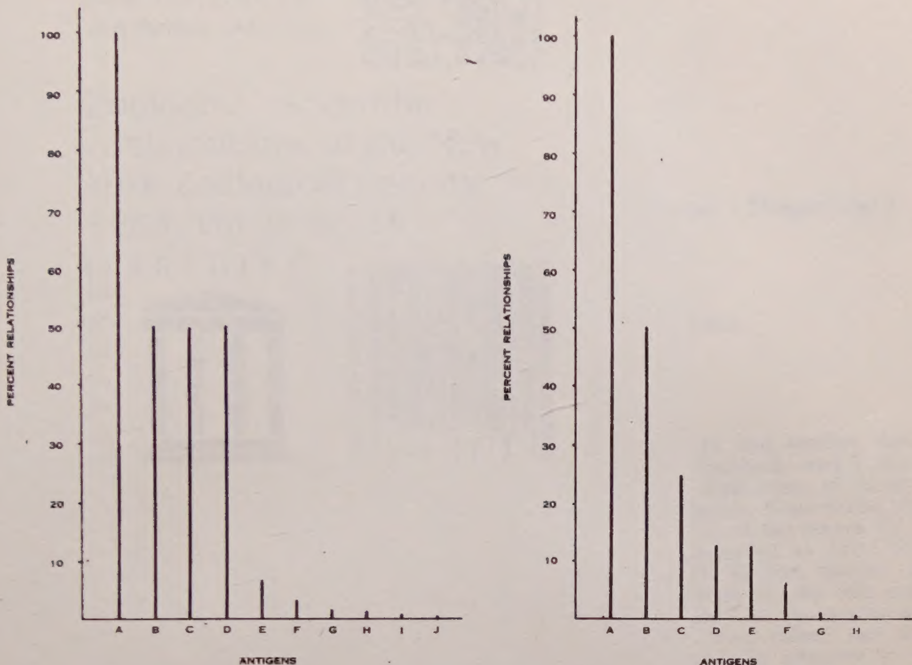
BOYDEN, ALAN A.
1942. Systematic serology: A critical appreciation. *Physiol. Zool.*, Vol. 15, No. 2.

BOYDEN, A. A. AND NOBLE, G. K.
1933. The relationships of some common amphibians as determined by serological study. *Amer. Museum Novitates*, No. 606.

DEFALCO, R. J.
1940. A serological study of some avian relationships. Thesis presented to the Graduate Faculty of Rutgers University. Published in part in *Biol. Bull.*, 1942, 83: 205-218.

GARSTANG, W.
1931. The phyletic classification of Teleostei *Proc. Leeds Phil. and Lit. Soc.* Vol, 2 (Sci. Sect.)

KOCH, F. C. AND MCMEEKIN, T. L.
1924. A new direct nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. *J. Amer. Chem. Soc.*, 46, 2066.



TEXT-FIG. 7. The reaction values by the ring test when the antiformolized pickerel and black bass antisera were tested with a number of antigens. It will be seen that the ring test brought in more distant relatives than could be shown with these same sera by the photron'er, but in the more closely related species of the Centrarchidae, the differentiation shown with the photron'er was not paralleled by the ring test.

Antiblack Bass II (Fo)
× A — Black Bass (Fo)
B — L.M. Bass (Fo)
C — B.G. Sunfish (Fo)
D — Rock Bass (Fo)
E — Perch (Fo)
F — Pickerel (Fo)
G — Dolphin (Fo)
H — Brook Trout (Fo)
I — Barracuda (Fo)
J — Kingfish (Fo)

Antipickerel II (Fo)
× A — Pickerel (Fo)
B — Perch (Fo)
C — B.G. Sunfish (Fo)
D — L.M. Bass (Fo)
E — Black Bass (Fo)
F — Rock Bass (Fo)
G — Barracuda (Fo)
H — Dolphin (Fo)

KRAUS, R.
1897. Uber specifische Reactionen in keimfreien Filtraten aus Cholera, Typhus und Pest bouillon Culturen erzeugt durch homologes serum. Wiener klin. Wochen., No. 32, 736.

LANDSTEINER, K.
1936. The specificity of serological reactions. Charles G. Thomas Co., Baltimore, Maryland.

LIBBY, R. L.
1938. The photronreflectometer, an instrument for the measurement of turbidity systems. *J. Immunol.,* 34, 71.

MEZ, C. AND ZIEGENSPECK, H.
1926. Zur theorie der serodiagnostik. *Bot. Arch.,* 13, 483.

NUTTALL, G. H. F.
1904. Blood immunity and blood relationships. Cambridge University Press.

WELLS, H. G.
1926. The chemical aspects of immunity. Chemical Catalogue Company, Baltimore, Maryland.

WOLFE, H. R.
1935. The effects of injection methods on the species specificity of serum precipitins. *J. Immunol.,* 29, 1.